AQUA-627747; No of Pages 11

Available online at www.sciencedirect.com

ScienceDirect

Aquaculture

ELSEVIER

Aquaculture xx (2007) xxx–xxx

www.elsevier.com/locate/aqua-online

Interleukin-10 expression by real-time PCR and homology modelling analysis in the European sea bass (*Dicentrarchus Labrax* L.)

Francesco Buonocore [a,*], Elisa Randelli, Steve Bird [b], Chris J. Secombes [b], Angelo Facchiano [c], Susan Costantini [c], Giuseppe Scapigliati [a]

[a] *Dipartimento di Scienze Ambientali, Università della Tuscia, 01100 Viterbo, Italy*
[b] *Scottish Fish Immunology Research Centre, Aberdeen University, AB24 2TZ Aberdeen, UK*
[c] *CNR, Istituto di Scienze dell'Alimentazione, I-83100 Avellino, Italy*

Received 25 January 2007; received in revised form 31 May 2007; accepted 31 May 2007

Abstract

In this paper we investigate the interleukin-10 (IL-10) expression and 3D modelling of the European sea bass (*Dicentrarchus labrax* L.). IL-10 is a regulatory cytokine that has been intensively studied in mammals and has been found mainly involved in the suppression or deactivation of activated immune responses. The full-length sea bass IL-10 cDNA consists of 1015 bp and is translated in a predicted molecule of 187 amino acids. A multiple alignment of this peptide with other known sequences showed the conservation of fundamental IL-10 family characteristics. Expression analysis by real-time PCR in control fish showed a high basal expression in the head kidney (HK), followed by gut and brain. *In vitro* treatment of HK leucocytes with LPS showed an intense increase of IL-10 expression at 4 h and a significant decrease at 24 h, with PHA-L no differences were evidenced. A 3D model for sea bass IL-10 was obtained by accurate homology procedures and putative interaction residues with the IL-10 receptor described. The results suggested that sea bass IL-10 could be involved in anti-inflammatory responses, but further experiments are needed to assess its importance in response to pathogenic agents, vaccinations and immunostimulants.

Keywords: Interleukin-10 (IL-10); European sea bass; *Dicentrarchus labrax*; Expression analysis; Real-time PCR; 3D modelling

1. Introduction

Cytokines are potent mediators of immune responses in jawed vertebrates, and in recent years a number of teleost cytokine genes with homology to mammalian genes have been discovered (Scapigliati et al., 2006a,b). Interleukin-10 (IL-10) is a regulatory cytokine, extensively studied in mammals, mainly involved in the suppression or deactivation of activated immune responses (Moore et al., 1993, 2001; Mosmann, 1994). IL-10 is produced by macrophages (Barnes et al., 1992) and by the T-helper 2 (Th_2) and T-regulatory (T_R) cell subsets (Mosmann and Moore, 1991; Street and Mosmann, 1991; Ding et al., 2003). It plays an important role in inflammation (Bogdan et al., 1991) by inhibiting some activities of macrophages (Fiorentino et al., 1991; Cunha et al., 1992), and certain cytokine production (de Waal Malefyt et al., 1993). IL-10 exerts its biological activity as an homodimer formed by two non-covalently linked

* Corresponding author. Dipartimento di Scienze Ambientali, Università della Tuscia, 01100 Viterbo, Italy. Tel.: +39 0761 357644; fax: +39 0761 357179.
E-mail address: fbuono@unitus.it (F. Buonocore).

0044-8486/$ - see front matter © 2007 Published by Elsevier B.V.
doi:10.1016/j.aquaculture.2007.05.040

Please cite this article as: Buonocore, F. et al. Interleukin-10 expression by real-time PCR and homology modelling analysis in the European sea bass (*Dicentrarchus Labrax* L.). Aquaculture (2007), doi:10.1016/j.aquaculture.2007.05.040

monomers (Tan et al., 1993) and each IL-10 subunit is stabilised by two intra-chain disulfides bonds (Windsor et al., 1993). The IL-10 gene has been recently discovered in the *Fugu* genome (*Fugu rubripes*) (Zou et al., 2003) and the cDNA cloned in different fish species (Savan et al., 2003; Inoue et al., 2005; Zhang et al., 2005; Pinto et al., 2006). From these studies it has emerged that IL-10 in fish is an α-helical polypeptide with a deduced molecular size of 18 kDa, derived from a gene composed of 5 exons and with significant structural similarities with its homologs in mammals (Zou et al., 2003). Moreover, its expression was up-regulated following intra-peritoneal injection of UV-killed *Photobacterium damselae* ssp. *piscicida* and seems involved in the resolution of inflammatory responses (Pinto et al., 2006). Therefore, it is interesting to determine if we have in sea bass the presence of an IL-10 dependent signal pathway to counteract the actions of pro-inflammatory factors, especially for its implication in response to pathogenic agents, vaccinations and immunostimulants.

In this work we studied the IL-10 expression by real-time PCR in sea bass (*Dicentrarchus labrax*) to further assess its implication and important role during anti-inflammatory processes. Moreover, we investigated sea bass IL-10 3D structure by homology modelling and analysed the putative interaction residues with the IL-10 receptor.

2. Materials and methods

2.1. Fish and leucocytes

Outbred mixed sex sea bass (178±37 g) used in this work were purchased from a local fish farm (Nuova Azzurro, Civitavecchia, RM). All buffers and solutions used in handling fish cells were brought to 355 mOsm/kg with 2 M NaCl. Fish were lethally anaesthetised with 1 g/l of benzocaine (Sigma), and 2 ml of blood per fish was drawn from the caudal vein using HBSS-heparin. Whole blood from individual fish was washed twice in HBSS-heparin (Gibco), resuspended in 8 ml of the same solution and loaded over 1.04 and 1.07 g/cm^3 Percoll gradients as previously described (Scapigliati et al., 2001) to obtain peripheral blood leucocytes (PBL). After centrifugation (30 min at 840 *g*) at 4 °C, cells at the interface between the two densities were collected, washed twice with HBSS (10 min at 680 *g*) at 4 °C, and used. The following organs and tissues were removed and placed in cold HBSS: head kidney (HK), spleen, liver, gills, gut, thymus and brain. Cells were obtained by teasing organs (excluding HK, gills and gut) through a 100-μm nylon mesh in HBSS and collecting the pelleted cells after two washes at 680 *g* in cold PBS. For HK, gills and gut cells were loaded and centrifuged over Percoll gradients as above to obtain the leukocyte enriched fraction. Cell viability of leukocytes was determined by counting in a haemocytometer with Trypan blue.

2.2. Sea bass IL-10 cloning

Two primers (IL10FR and IL10RV, see Table 1) corresponding to highly conserved regions of known IL-10 genes were used for RT-PCR on total RNA extracted with Tripure (Roche) solution from juvenile sea bass (150 g of weight) gut cells obtained following the procedures described above. For cDNA synthesis, 1 μg of total RNA and 0.5 μg of random primers [pd(N)$_6$] were used in each reverse transcription reaction in a total volume of 50 μl. Reactions were conducted using the Mastercycler personal (Eppendorf). The cycling protocol was one cycle of 94 °C for 5 min, 35 cycles of 94 °C for 45 s, 55 °C for 45 s, 72 °C for 45 s, followed by one cycle of 72 °C for 10 min. PCR products (15 μl) were visualised on 1% (w/v) agarose gels containing ethidium bromide (10 ng/ml) using hyperladder IV (Bioline) as size marker. Controls for the presence of DNA contamination were performed using

t1.1 Table 1
t1.2 Oligonucleotides and primers used for cDNA cloning and real-time PCR

t1.3 Primer	Sequence	Function
t1.4 IL10FR	5′-TGCTGCTCCTTCGTGGAGGGCTTCCC-3′	Cloning initial sea bass IL-10 sequence
t1.5 IL10RV	5′-CCAGCTCCCCCATGGCTTTATA-3′	“
t1.6 Oligo-dT adaptor primer	5′-CTCGAGATCGATGCGGCCGCT$_{15}$-3′	3′ RACE-PCR
t1.7 SB10FR	5′-CAGATGGAGAGTAAAGGTC-3′	“
t1.8 Oligo-dG	5′-GGGGGGIGGGIIGGGIIG-3′	5′ RACE-PCR
t1.9 SB10RV	5′-GAGTGATGTCACCAGATG-3′	“
t1.10 RTACTFR2	5′-ATGTACGTTGCCATCC-3′	Real-time PCR for sea bass β-actin
t1.11 RTACTRV2	5′-GAGATGCCACGCTCTC-3′	“
t1.12 RTIL10SBAFR	5′-ACCCCGTTCGCTTGCCA-3′	Real-time PCR for sea bass IL-10
t1.13 RTIL10SBARV	5′-CATCTGGTGACATCACTC-3′	“

t1.14 I in the oligo-dG primer is referred to inosine.

Please cite this article as: Buonocore, F. et al. Interleukin-10 expression by real-time PCR and homology modelling analysis in the European sea bass (*Dicentrarchus Labrax* L.). Aquaculture (2007), doi:10.1016/j.aquaculture.2007.05.040

the RNA samples as template. DNA amplified by PCR was purified using the QIAquick Gel Extraction Kit (QIAgen), inserted into the pGEM-T Easy vector (Promega) and transfected into competent JM109 *Escherichia coli* cells. Plasmid DNA from at least four independent clones was purified using the Wizard Plus SV Minipreps DNA Purification System (Promega) and sequenced using MWG DNA Sequencing Services. Sequences generated were analysed for similarity with other known sequences using the FASTA (Pearson and Lipman, 1988) and BLAST (Altschul et al., 1990) programs and multiple alignments were made with CLUSTAL W (Thompson et al., 1994).

Further primers were designed against the initial sea bass IL-10 sequence for 5′- and 3′-rapid amplification of cDNA ends (RACE)-PCR (SB10FR and SB10RV, see Table 1). cDNA was synthesised from total gut RNA with the First-strand cDNA Synthesis kit (Amersham Pharmacia) following the manufacturer's instructions. For 3′ RACE-PCR, cDNA was transcribed using an oligo-dT adaptor primer (see Table 1). PCR was performed with the SB10FR primer and the oligo-dT adaptor primer. For 5′ RACE-PCR, cDNA was transcribed from total RNA using the oligo-dT primer, treated with *E. coli* RNase H (Promega), purified using a PCR Purification Kit (QIagen), and tailed with poly(C) at the 5′ end with terminal deoxynucleotidyl transferase (TdT, Promega). PCR was performed with SB10RV primer and an oligo-dG primer (see Table 1). Sequencing and similarity searches were as described above.

The obtained cDNA sequences were analysed for the presence of a signal peptide, using SignalP software (Nielsen et al., 1997), and for N- (with the NetNGlyc 1.0 Server) and O-linked glycosylation sites (Julenius et al., 2005). A phylogenetic tree was constructed by the "neighbour-joining" method using CLUSTAL W (Thompson et al., 1994) and PHYLIP packages (Felsenstein, 1985; Saitou and Nei, 1987) on full-length amino acid sequences and bootstrapped 10000 times (Felsenstein, 1985).

2.3. Basal IL-10 expression analysis

To study the IL-10 basal expression, five sea bass (178± 37 g) were sampled and leucocytes from different tissues obtained as described above. Total RNA was isolated from each tissue separately with Tripure (Roche) following the manufacturer's instructions, resuspended in DEPC treated water and used for real-time quantitative PCR without pooling the samples coming from the different fishes. Controls for the presence of DNA contamination were performed using the RNA samples as template and using β-actin primers that bracket an intron.

For reverse transcription, the BioScript RNase H minus (Bioline) enzyme was used with the following protocol: 2 μg of total RNA was mixed with 1 μl of random hexamer (0.2 μg/μl; Amersham Pharmacia) and nuclease free water was added to a final volume of 12 μl. This mixture was incubated at 70 °C for 5 min and then cooled on ice. Successively, 0.4 μl of a reaction mix containing 100 mM dNTPs (25 mM each; Promega), 4 μl of 5X Reaction buffer, nuclease free water to a final volume of 19.75 μl and 0.25 μl of BioScript at 200 u/μl were added and the solution incubated at 25 °C for 10 min and then at 37 °C for 60 min. Finally, the reaction was stopped by heating at 70 °C for 10 min.

The expression level of IL-10 was determined with a Mx3000P™ real-time PCR system (Stratagene) equipped with version 2.02 software and using the Brilliant SYBR Green Q-PCR Master Mix (Stratagene) following the manufacturer's instructions, with ROX as internal passive reference dye. The reference dye is not reactive during real-time PCR and therefore can be used to normalize slight differences in the volume of the added real-time PCR reaction, transparency of the plastic caps and other sources of well-to-well differences. Specific PCR primers were designed for the amplification of about 200 bp products from both IL-10 and β-actin, used as a house-keeping gene. The primers were: RTIL10SBAFR and RTIL10SBARV, RTACTFR2 and RTACTRV2, respectively (see Table 1). Approximately 20 ng of cDNA template was used in each PCR reaction. The PCR conditions were 95 °C for 10 min, followed by 35 cycles of 95 °C for 45 s, 52 °C for 45 s and 72 °C for 45 s. Triplicate reactions were performed for each template cDNA and the template was replaced with water in all blank control reactions. Each run was terminated with a melting curve analysis which resulted in a melting peak profile specific for the amplified target DNA. The analysis was carried out using the endpoints method option of the Mx3000P™ software that causes the collection of the fluorescence data at the end of each extension stage of amplification. A relative quantitation has been performed, comparing the levels of the target transcript (IL-10) to a reference transcript (calibrator, the tissue with the lowest IL-10 expression, in this case the thymus). The expression level of the gene of interest (IL-10) in the calibrator is defined as 1.0. A normalizer target (β-actin) is included to correct for differences in total cDNA input between samples. The quantitative experiment is based on threshold cycle (Ct) determination, defined as the cycle at which a statistically-significant increase in fluorescence (above background signal) is detected. The results are expressed as the mean±SD of the results obtained from the five considered fishes. The real-time PCR products from the

Please cite this article as: Buonocore, F. et al. Interleukin-10 expression by real-time PCR and homology modelling analysis in the European sea bass (*Dicentrarchus Labrax* L.). Aquaculture (2007), doi:10.1016/j.aquaculture.2007.05.040

different tissues were examined successively by agarose gel electrophoresis to investigate their specificity and size.

2.4. In vitro expression analysis

The *in vitro* expression of IL-10 was studied using different stimulating conditions on HK leucocytes from five fishes as above.

In the first stimulation, HK leucocytes were adjusted to 1×10^5 cells/ml and incubated at 18 °C for 4 h and 24 h with 5 μg/ml of lipopolysaccharide (LPS from *E. coli* 0127:B8, Sigma) in PBS or with PBS for the same time points (control).

In the second stimulation, HK leucocytes were adjusted to 1×10^5 cells/ml and incubated at 18 °C for 4 h and 24 h with 1 μg/ml of lectin from *Phaseolus vulgaris* Leucoagglutinin (PHA-L from Sigma) in PBS or with PBS for the same time points (control).

Total RNA was isolated with Tripure (Roche) following the manufacturer's instructions, resuspended in DEPC treated water and used for real-time quantitative PCR without pooling the samples coming from the different fishes. The primers used are described in Table 1. Real-time PCR conditions were as described above, except that the calibrator for this experiment was the 0 h control.

Data were expressed as the mean±SD of the results obtained from the five considered fishes and the differences from the control at the same time have been considered significant if $p<0.05$ using the standard student *t* test to analyse the significance.

2.5. Protein modeling

Protein sequences used in modelling refer to the following EMBL entries: seabass IL-10, AM268529; human IL-10, M57627 (UniProt: P22301). The three-dimensional model of the sea bass IL-10 was created according to the homology modelling strategy and using the template model of human IL-10 (PDB code: 1ILK) (Zdanov et al., 1995).

As the sequence identities between the sea bass protein and the human homologous template were lower than 30%, we used an accurate procedure to search for the best alignment of sequences, already used and described in our previous works (Facchiano et al., 2001; Marabotti et al., 2004; Scapigliati et al., 2004; Costantini et al., 2005; Buonocore et al., 2006; Facchiano et al., 2006) and also in agreement with the rules recently reviewed (Wallner and Elofsson, 2005) to improve the quality of the modeling results. The search for sequence similarity within databases was performed with the BLAST program (Altschul et al., 1990). The alignment of the protein sequences was made with CLUSTALW program (Thompson et al., 1994) and a few manual refinements were added to account for the position of secondary structures. The program MODELLER (Sali and Blundell, 1993) implemented in the Quanta molecular simulation package (Accelrys, San Diego, CA) was used to build 10 full-atom models by setting 4.0 Angstrom as RMS deviation among the structures of the templates and fully optimized models, with multiple cycles of refinement with conjugate gradient minimization and molecular dynamics with simulated annealing. To select the best model among those obtained, PROCHECK program (Laskowski et al., 1993) was used to evaluate the stereochemical quality of the models and their structural packing quality and ProsaII program (Sippl, 1993) to check the fitness of sequence to structure and to assign a scoring function. Secondary structures were assigned by the DSSP program (Kabsch and Sander, 1983). Search for structural classification was performed on CATH database (Orengo et al., 1997; Pearl et al., 2000).

The atomic coordinates of the human dimer were built operating the symmetry transformations on the atoms of monomer reported in the PDB file by using the tool "Build Crystallographic Symmetry" implemented in SwissPdb-Viewer software (Guex and Peitsch, 1997). The "Protein– Protein Interaction Server" (Jones and Thornton, 1996) and the program NACCESS (Hubbard et al., 1991) were used to identify the amino acids at the protein–receptor interface in the crystallographic complexes. Molecular superimposition, RMSD values and figures were obtained with the InsightII package (Accelrys, Inc., San Diego, CA, USA).

3. Results

3.1. Sea bass IL-10 cloning

PCR with primers IL10FR and IL10RV gave a product of the expected size (406 bp) that when sequenced had good homology for other known IL-10 sequences (data not shown). 5′-RACE-PCR performed with SB10RV (based on the initial 406 bp sequence) and oligo-dG primer gave a product of about 530 bp that contained the 5′-end of the gene. 3′-RACE-PCR performed with SB10FR (based on the initial 406 bp sequence) and oligo-dT adaptor primer gave a product of about 400 bp that contained the 3′-end of the gene. The full-length nucleotide sequence (EMBL accession number AM268529) is comprised of 1015 bp obtained from the three overlapping products. It was translated in one reading frame to give the entire IL-10 molecule containing 187 amino acids, with a predicted 22 amino acid signal peptide, a 143 bp 5′-UTR and a 313 bp

Please cite this article as: Buonocore, F. et al. Interleukin-10 expression by real-time PCR and homology modelling analysis in the European sea bass (*Dicentrarchus Labrax* L.). Aquaculture (2007), doi:10.1016/j.aquaculture.2007.05.040

3′-UTR. Finally, the 3′-UTR contained a polyadenylation signal (AATAAA) 17 bp upstream of the poly(A) tail and three mRNA instability motifs (ATTTA).

A multiple alignment of the predicted translation of the IL-10 sea bass molecule with other known IL-10 sequences was assembled (Fig. 1) to investigate the conservation of

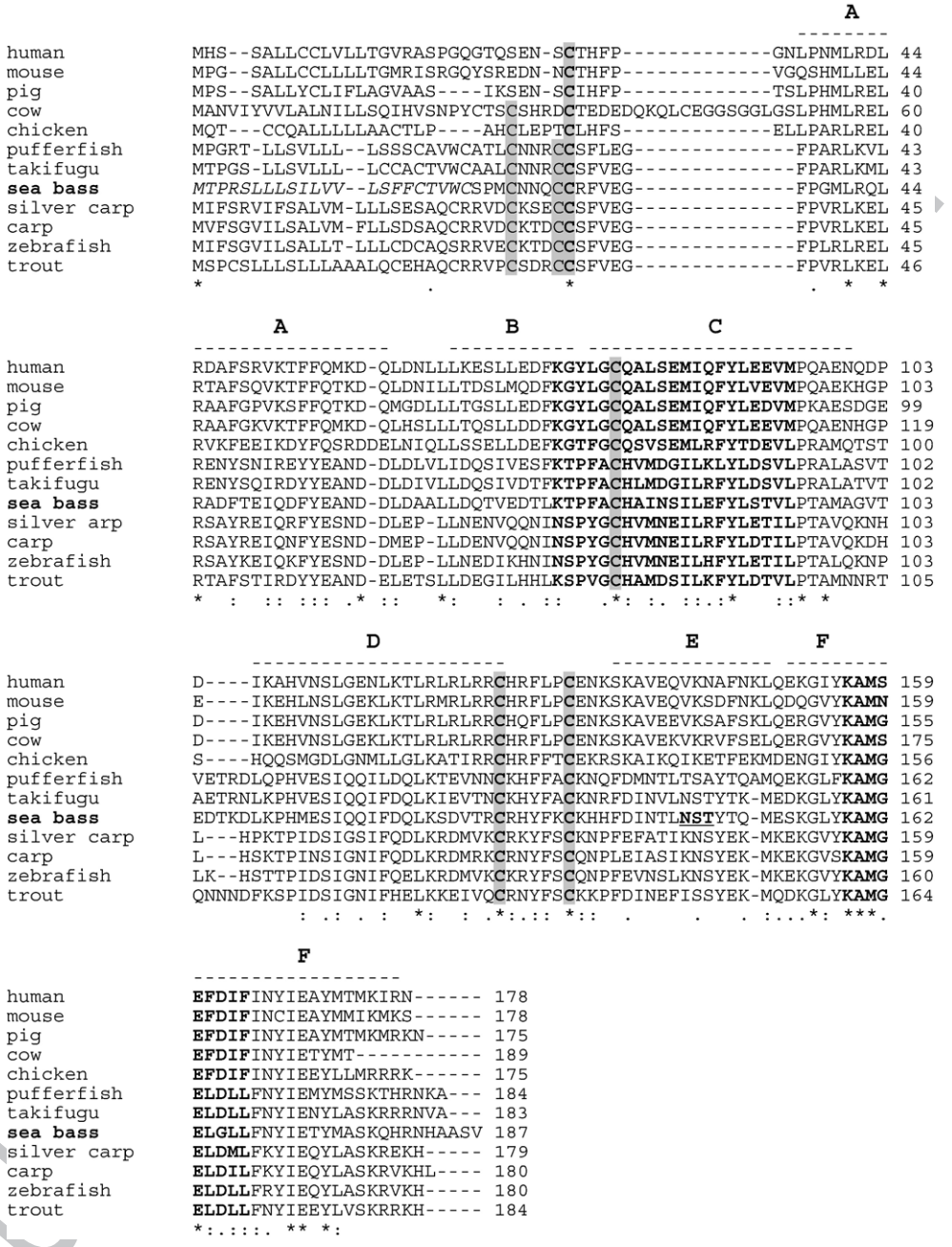

Fig. 1. Alignment of the predicted sea bass IL-10 amino acid sequence with other known IL-10 molecules. Identical (*) and similar (. or :) residues identified by CLUSTAL W are indicated. The conserved cysteine residues and the IL-10 family signature motifs are gray and bolded. The six α-helical domains (A–F) predicted in human IL-10 are marked by hyphens. In the sea bass IL-10 sequence the predicted signal peptide is in italics and the putative *N*-glycosilation site is underlined and bolded. Accession numbers: *Homo sapiens* (human) U16720; *Mus musculus* (house mouse) M37897; *Sus scrofa* (pig) L20001; *Bos bovis* (cow) AY186733; *Gallus gallus* (chicken) NP_001004414; *Tetraodon nigroviridis* (spotted green puffer fish) AJ544898; *Takifugu rubripes* (Fugu rubripes) AJ539537; *Hypophthalmichthys molitrix* (silver carp) DQ058295; *Cyprinus carpio* (common carp) AB110780; *Danio rerio* (zebrafish) AY887900; *Oncorhynchus mykiss* (rainbow trout) AB118099.

Please cite this article as: Buonocore, F. et al. Interleukin-10 expression by real-time PCR and homology modelling analysis in the European sea bass (*Dicentrarchus Labrax* L.). Aquaculture (2007), doi:10.1016/j.aquaculture.2007.05.040

amino acid residues involved in the structural domains found within IL-10s. The putative crystal structure deduced from human recombinant IL-10 showed the presence of six helices (A–F, Fig. 1) with approximately 85% of the residues in α-helical conformation (Zdanov et al., 1995) and revealed that the molecule is likely to exist in solution as a tight dimer made of two inter-penetrating subunits. The helices with the highest degree of homology between the human and fish sequences are the C-helix and the F-helix that contain the IL-10 family signature motifs (Inoue et al., 2005). Moreover, it is noteworthy to observe that some of the amino acid residues found in helices are well conserved only between the fish sequences, especially in the A, C and F stretches.

The sea bass IL-10 molecule contains four cysteine residues (Cys-30, 80, 130 and 136) that are conserved in all IL-10 vertebrate sequences showed in the alignment, although two extra-cysteine residues (Cys-26 and 31) are present in the N-terminal region and conserved in all fish sequences but not in mammals. The structure of human IL-10 is stabilized by two intra-molecular disulfide bridges that join helices A and C to helix D (Windsor et al., 1993; Zdanov et al., 1995) and the involved cysteine residues are the ones conserved in all IL-10 sequences. A unique putative N-glycosylation site has been found in the sea bass IL-10 sequence (Asn146, Ser147, Thr148) in the same position found in the *Fugu* molecule but different from the one found in trout, although glycosylation seems not to be required for biological activity.

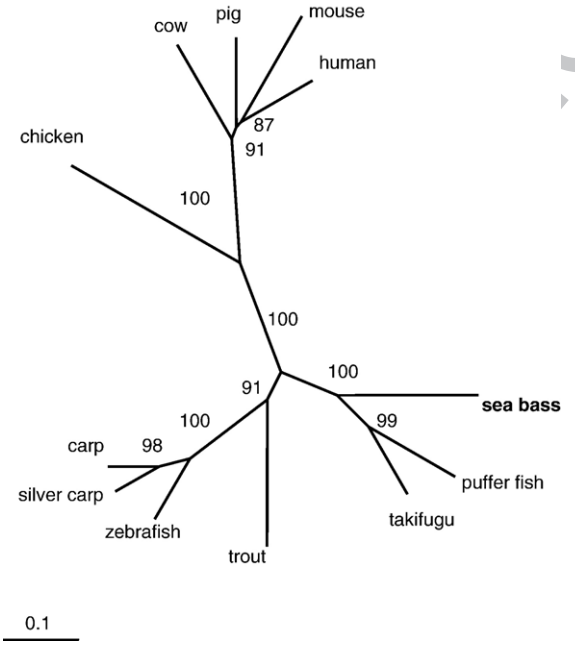

Fig. 2. Unrooted phylogenetic tree showing the relationship between sea bass IL-10 with other known IL-10 molecules. The tree was constructed by the "neighbour-joining" method and was bootstrapped 10,000 times. The bar (0.1) indicates the genetic distance.

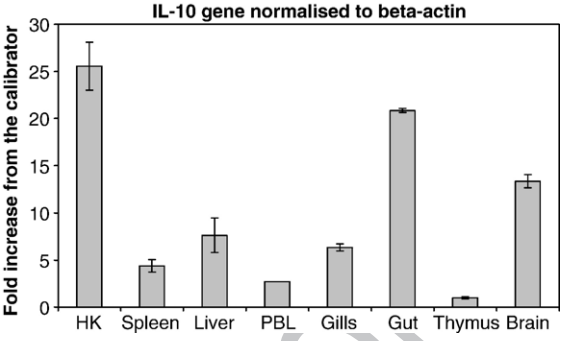

Fig. 3. IL-10 basal expression in different tissues. IL-10 mRNA levels were expressed as a ratio relative to β-actin levels in the same samples after real-time PCR analysis using the tissue with the lowest expression as calibrator.

Phylogenetic analysis (Fig. 2) conducted using amino acid sequences showed that sea bass IL-10 grouped with other known teleost sequences close to *Tetraodon* and *Fugu*. Moreover, the presence of three different clusters formed by the mammalian, the avian and the fish IL-10 sequences was quite evident. The positions in the tree were all supported by high bootstrapping values.

3.2. IL-10 basal expression levels

The expression analysis of IL-10 in organs and tissues of un-stimulated sea bass is shown in Fig. 3. Real-time PCR products were loaded on agarose gels to exclude the formation of non-specific amplicons and single bands of the expected sizes were obtained. Moreover, to take into consideration the individual genetic variability five different fish were sampled and analysed separately. The highest IL-10 expression was detected in HK, followed by gut. Lower IL-10 mRNA levels were observed in brain, liver, gills, and spleen. PBL and thymus showed the lowest expression levels.

3.3. In vitro expression analysis

To investigate whether IL-10 expression level could be modulated with LPS and PHA-L, *in vitro* stimulation of HK leucocytes for a short (4 h) and a longer (24 h) time was studied, RNA was extracted from the HK leucocytes of five individuals analysed separately. Real-time PCR products were loaded on agarose gels and single bands of the expected sizes were obtained as above. The results are shown in Fig. 4. Using LPS (Fig. 4A), a dramatic increase of IL-10 expression was detected at 4 h ($p<0.05$), whereas at 24 h ($p<0.05$) it

Please cite this article as: Buonocore, F. et al. Interleukin-10 expression by real-time PCR and homology modelling analysis in the European sea bass (*Dicentrarchus Labrax* L.). Aquaculture (2007), doi:10.1016/j.aquaculture.2007.05.040

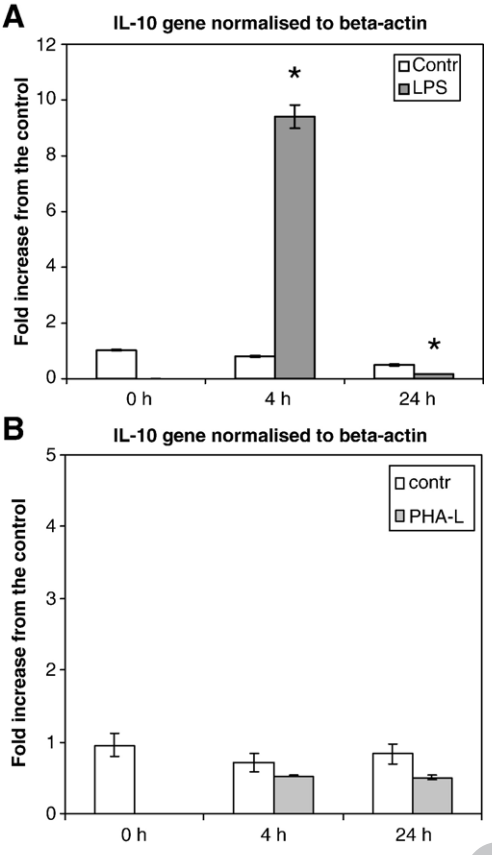

Fig. 4. *In vitro* sea bass IL-10 expression analysis. (A) LPS: IL-10 mRNA levels expressed as a ratio relative to β-actin levels in the same samples after real-time PCR analysis of HK leucocytes stimulated with PBS (control) and with 5 μg/ml LPS for 4 and 24 h and normalised against the non-stimulated controls. (B) PHA: IL-10 mRNA levels expressed as a ratio relative to β-actin levels in the same samples after real-time PCR analysis of HK leucocytes stimulated with PBS (control) and with 1 μg/ml PHA-L for 4 and 24 h and normalised against the non-stimulated controls. Controls for 4 and 24 h of incubation without LPS and PHA-L are also shown in the graphs. Data were expressed as the mean ± SD and asterisks indicates when $p \leq 0.05$ with respect to their control.

was lower than the control. When using PHA for *in vitro* treatment (Fig. 4B), a decrease was observed both at 4 h and 24 h, although being not statistically significant.

3.4. 3D modelling of sea bass IL-10

The sequence of sea bass IL-10 has been analyzed with the BLAST program to find similar sequences in databases and perform structure predictions. The search in the non-redundant database has evidenced that this protein is significantly similar (*E*-value less than 10^{-12}) to 34 protein sequences, from different organisms, defined as "IL-10" (data not shown). The percentage of sequence identity between IL-10 in sea bass and the other sequences ranged from 67% to 44% for teleost fish sequences, and from 30% to 27% for mammal sequences. The search also found the experimental three-dimensional structure of human IL-10 (PDB code: 1ILK) (Zdanov et al., 1995), that could be used for comparative modeling. The BLAST pairwise alignment between sea bass and human sequences evidenced amino acid identity lower than the threshold value of 30%. This level of sequence identity requires a careful procedure to build a 3D model of the protein by comparative modeling.

Multiple alignment of the IL-10 sequences was performed with the CLUSTALW program and starting from this alignment (Fig. 5), we created ten structural models for the sea bass IL-10 28–187 region. The best model was chosen evaluating the scoring functions with ProsaII program (Sippl, 1993). The stereo chemical quality in human and sea bass models was evaluated using the PROCHECK program (Laskowski et al., 1993) and the percentage of residues in most favoured regions was 95.0% in human structure and 94.6% in the chosen model for sea bass IL-10. The quaternary structure of IL-10 dimer was assembled by superimposing two equal sea bass (monomers) chains to those in human dimer. In Fig. 6

Fig. 5. Primary and secondary structures of sea bass IL-10, aligned with human IL-10 as for the modelling procedure using CLUSTAL W. The interaction residues of IL-10 with IL-10R are reported in bold. The cysteine is evidenced in bold/italics. The six helices found in the IL-10 structures are marked with arrows. Alpha helices are indicated with continuous line boxes and 3_{10} helices with dashed line boxes.

Please cite this article as: Buonocore, F. et al. Interleukin-10 expression by real-time PCR and homology modelling analysis in the European sea bass (*Dicentrarchus Labrax* L.). Aquaculture (2007), doi:10.1016/j.aquaculture.2007.05.040

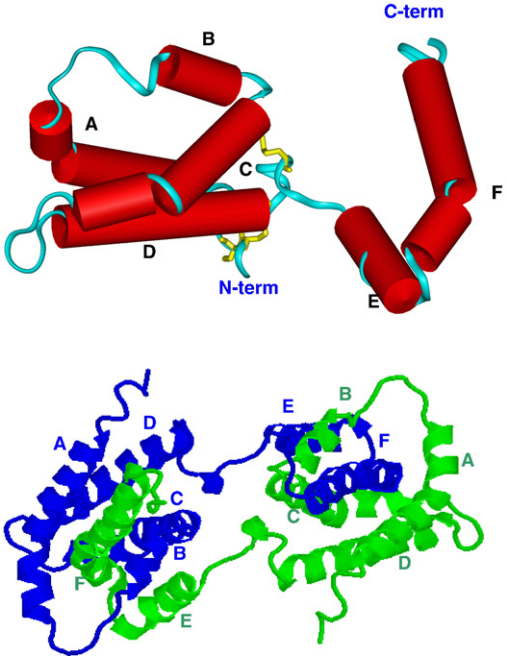

Fig. 6. The monomer and the dimer of seabass IL-10. (a) Backbone ribbon of monomer of sea bass IL-10 obtained by homology modelling with evidenced the secondary structure topology (*i.e.* red cylinders represent alpha helices). Amino and carboxy terminal ends are indicated. Two disulfide bridges are indicated with yellow sticks. (b) Ribbon diagram of the dimer of sea bass IL-10 with the subunits shown in green and blue. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

we reported the models of the monomer and the dimer of sea bass IL-10 (PDB code 2IFK). About 70% of the residues in each monomer assumes an alpha-helical conformation. Each chain consists of two separate domains, one in the N-terminal region composed of four α-helices (A–D), and the other in the C-terminal region composed of two α-helices (E and F) in agreement to the structural classification reported by CATH database (Orengo et al., 1997; Pearl et al., 2000) for the reference structure of human IL-10. The internal structure of two domains is quite rigid but the inter-domain junction between helices D and E, bringing the two domains together, is potentially flexible. Two intra-molecular disulfide bridges, Cys30-Cys130 and Cys79-Cys136 stabilize each monomer. A potential N-linked glycosylation site, that in human IL-10 is located in the loop between helices D and E and is conserved in all mammalian and also in carp, is not present in sea bass.

4. Discussion

Our knowledge of cytokines present in teleost fish has dramatically increased in recent years and this animal group is becoming an excellent model among ectothermic vertebrates for studies of their effects on the immune system (Bird et al., 2006; Scapigliati et al., 2006a,b). Despite this impressive advancement, the knowledge on the *in vivo* and *in vitro* biological activity of fish cytokines is still meager, mainly due to the lack of recombinant peptides or antibodies. To further investigate the evolutionary biology of cytokines in teleost fish, and having molecular probes for some immunoregulatory genes of sea bass, in this work we considered IL-10 as a putative good candidate to study T-cell activities.

Sea bass IL-10 molecule is not highly homologous to its mammalian counterparts (30% amino acid identity with human IL-10), although the residues and motifs essential for the secondary and tertiary structures are well conserved. The four cysteine residues involved in the formation of intra-molecular disulphide bridges in human IL-10 are conserved, whereas two additional cysteines in the N-terminal region of the molecule are present only in fish sequences. Whether these two residues are important for the IL-10 structure remains to be elucidated. The nucleotide sequence of sea bass IL-10 contains three mRNA instability motifs (ATTTA) which are known to influence mRNA half-life and translational efficiency. This motif, that is believed to play a role in IL-10 regulation (Brown et al., 1996), is present in all vertebrate IL-10 3′-UTR except in rainbow trout (Inoue et al., 2005). The obtained phylogenetic tree shows that sea bass IL-10 grouped in a branch with other teleost sequences, with *Tetraodon* and *Fugu* as the most closely related.

Real-time PCR analysis detected high levels of constitutive IL-10 expression in HK and gut, as already observed in common carp (Savan et al., 2003). However, this feature appears to be quite variable between fish species, since a very low constitutive expression in all tissues sampled was found in *Fugu* (Zou et al., 2003) and zebrafish (Zhang et al., 2005), whereas in rainbow trout an intense expression is only in the gills (Inoue et al., 2005). It should be noted that expression of IL-10 is notably elevated in the gut, and we have previously shown this mucosal tissue be particularly rich in T-cells (Abelli et al., 1997). Moreover, the IL-10 expression level is very low in the thymus and this could resemble what happens in rodents and human where the naturally occurring CD4+ CD25+ T regulatory cells (Tregs) that emerge directly from this organ do not produce high IL-10 levels, whereas the T regulatory type 1 (Tr1) cells, induced by antigen stimulation via an IL-10 dependent process, show high IL-10 and transforming growth factor β (TGF-β) (Roncarolo et al., 2006). These observations could be

Please cite this article as: Buonocore, F. et al. Interleukin-10 expression by real-time PCR and homology modelling analysis in the European sea bass (*Dicentrarchus Labrax* L.). Aquaculture (2007), doi:10.1016/j.aquaculture.2007.05.040

particularly important when sea bass recombinant IL-10 will become available to investigate in fish its activities, such as the potent effects this cytokine has on human intestinal T-cells (Braunstein et al., 1997).

In close agreement with previous studies, the *in vitro* stimulation with LPS induced in sea bass HK a high IL-10 expression after 4 h, as observed in trout (Inoue et al., 2005), zebrafish (Zhang et al., 2005), carp (Savan et al., 2003), and in human monocytes (Gallagher et al., 2000). After 24 h of LPS stimulation, a dramatic decrease was observed in sea bass in agreement with results in common carp (Savan et al., 2003), and may suggest that teleost IL-10 may also play a role in the fast processes related to inflammation. However, in a recently published paper, Gonzales et al. (2007) analysed the carp skin inflammatory response after a mechanical injury that mimic infection with ectoparasites and found that no up-regulation of IL-10 was found at time points post-up-regulation of IL-1β even if they have data only until 24 h post-injury.

On the other hand, the *in vitro* treatment of HK leucocytes with the cell mitogen agent PHA-L showed a decrease in IL-10 expression both at 4 h and 24 h post-stimulation although being not statistically significant. This first observation is in contrast to data in PHA-activated chicken thymocytes (Rothwell et al., 2004) and human PBL (Lagrelius et al., 2006). It remains to be elucidated if this difference could be attributable to the anatomical difference between examined tissues, or to different capability of IL-10 regulation between fishes and other vertebrates.

Sea bass IL-10 3D structure has been predicted by homology modelling procedures and it is resulted as a symmetric homodimer, topologically similar to the structure of interferon-γ Each alpha-helical domain is made of two inter-penetrating subunits, forming a V-shaped structure. In fact, the chains of each subunit contribute to both halves of the dimer: helices E and F of one monomer form a distinctive six-helix domain with helices A′–D′ of the symmetry-related monomer. In addition, helices A, C, D, F′ and A′, C′, D′, F of each domain form a classical left-handed four-helix bundle, found in all crystal structures of the helical cytokine family. The presence of gaps in the alignment made it difficult a complete structural comparison of sea bass IL-10 with the human template. Anyway, superimposition of structurally conserved regions of two structures gives RMSD values of 1.07 Å. This value indicates that, despite the low sequence identities, sea bass sequence is well compatible with human IL-10 structure, according with the energy profiles obtained with ProsaII program (data not shown). The comparison of secondary structures suggests that all helices are well conserved among two species, although few external residues can result added or excluded from the helices (see Fig. 5). The short 3_{10} helix, observed in human IL-10 between the helices D and E is present also in the sea bass protein. Also the bends, located in helices A, C and F, in the human structure, are conserved even if these are shorter in the sea bass model.

Moreover, we investigated the possible residues of interaction of sea bass IL-10 with its receptor on the basis of the complex between human IL-10 dimer and its receptor molecule (PDB code: 1J7V) (Josephson et al., 2001). The crystal structure of this complex consists of one IL-10 homodimer and two IL-10R1 molecules. Each receptor binds the identical two-fold related surfaces of

Table 2
Solvent exposure of IL-10 amino acids interacting with IL-10R in sea bass and human

Amino acids	% Solvent exposure in sea bass IL-10	Amino acids	% Solvent exposure in human IL-10
Helix A		*Helix A*	
PHE 37	**28.4**	**LEU 37**	**14.9**
PRO 38	67.1	PRO 38	62.6
GLY 39	**43.9**	**ASN 39**	**60.3**
LEU 41	34.6	LEU 41	42.4
ARG 42	**69.9**	**ARG 42**	**55.9**
ARG 45	63.5	ARG 45	67.5
ALA 46	37	ASP 46	31.2
PHE 48	47.9	PHE 48	48.4
THR 49	48.8	SER 49	58.6
GLN 52	45	LYS 52	40.9
ASP 53	62.3	THR 53	58.2
GLU 56	63.3	GLN 56	60.6
ALA 57	65.7	MET 57	72.3
Loop AB		*Loop AB*	
ASP 59	55.3	ASP 59	63.2
ASP 60	92.4	GLN 60	90.8
LEU 61	78.5	LEU 61	72.2
ASP 62	100.9	ASP 62	105
ALA 63	65.1	ASN 63	71.8
ALA 64	**74.1**	**LEU 64**	**61.2**
LEU 65	**93.6**	**LEU 65**	**78**
Helix F		*Helix F*	
LYS 159	52.7	LYS 156	53
GLY 162	**81.4**	**SER 159**	**70.8**
GLU 163	50	GLU 160	41
GLY 165	53.3	ASP 162	61.5
LEU 166	49	ILE 163	40.2
ASN 169	49.8	ASN 166	57.6
TYR 170	64.5	TYR 167	69.8
GLU 172	**44.4**	**GLU 169**	**56.1**
MET 175	55.8	MET 172	47.9
ALA 176	**45.3**	**THR 173**	**30.9**
GLN 179	73.1	ILE 176	69.8
HIS 180	56.7	ARG 177	66

The amino acid in bold showed significant percentage differences.

Please cite this article as: Buonocore, F. et al. Interleukin-10 expression by real-time PCR and homology modelling analysis in the European sea bass (*Dicentrarchus Labrax* L.). Aquaculture (2007), doi:10.1016/j.aquaculture.2007.05.040

IL-10. Residues in the interface are donated from two peptide segments. Moreover, they cluster in two structurally distinct interaction surface, Ia and Ib. Site Ia is centered on the bend in the helix F′ and includes the AB loop, while the site Ib is located near the N terminus of helix A and the C terminus of helix F′. Being the residues of interaction located in the bends present in helices A and F, their different length in human and sea bass models could indicate a possible difference in the complex between this two species. The percentage of solvent exposure for each residue of interaction in human IL-10 and for the related sea bass residues identified on the basis of the alignment was evaluated and the results are reported in Table 2. Some differences can be appreciated in the solvent exposure values of various amino acids (shown in bold during the table) and this should strengthen the possibility that the complex is stabilised in a slight different way in sea bass with respect to human. The knowledge of the putative interaction residues between IL-10 and its receptor could be useful to produce synthetic peptides that, designed on the basis of the structural prediction, should block the site of interaction and provide the possibility to study what happens when the IL-10 signal could not be transduced.

In conclusion, the results we obtained in this work could be useful to increase the knowledge about fish IL-10 even if, so far, a clear anti-inflammatory function remains to be demonstrated.

Acknowledgements

Authors are indebted to Dr. C. Magugliani and Dr. E. Vela (Nuova Azzurro, Roma) for the supply of fish. The work was partially supported by the European Commission within the project IMAQUANIM (EC contract number FOOD-CT-2005-007103).

References

Abelli, L., Picchietti, S., Romano, N., Mastrolia, L., Scapigliati, G., 1997. Immunohistochemistry of gut-associated lymphoid tissue of the sea bass *Dicentrarchus labrax* (L.). Fish Shellfish Immunol. 7, 235–246.

Altschul, S.F., Gish, W., Miller, W., Myers, E., Lipman, D.J., 1990. Best local alignment search tool. J. Mol. Biol. 215, 403–410.

Barnes, P.F., Chatterjee, D., Abrams, J.S., Lu, S., Wang, E., Yamamura, M., Brennan, P.J., Modlin, R.L., 1992. Cytokine production induced by *Mycobacterium tuberculosis* lipoarabinomannan. Relationship to chemical structure. J. Immunol. 149, 541–547.

Bird, S., Zou, J., Secombes, C.J., 2006. Advances in fish cytokine biology give clues to the evolution of a complex network. Curr. Pharm. Des. 12, 3051–3069.

Bogdan, C., Vodovotz, Y., Nathan, C., 1991. Macrophage deactivation by interleukin 10. J. Exp. Med. 174, 1549–1555.

Braunstein, J., Qiao, L., Autschbach, F., Schurmann, G., Meuer, S., 1997. T cells of the human intestinal lamina propria are high producers of interleukin-10. Gut 41, 215–220.

Brown, C.Y., Lagnado, C.A., Vadas, M.A., Goodall, G.J., 1996. Differential regulation of the stability of cytokine mRNAs in lipopolysaccharide-activated blood monocytes in response to interleukin-10. J. Biol. Chem. 271, 20108–20112.

Buonocore, F., Randelli, E., Bird, S., Secombes, C.J., Costantini, S., Facchiano, A., Mazzini, M., Scapigliati, G., 2006. The CD8alpha from sea bass (*Dicentrarchus labrax* L.): cloning, expression and 3D modelling. Fish Shellfish Immunol. 20, 637–646.

Costantini, S., Colonna, G., Rossi, M., Facchiano, A.M., 2005. Modelling of HLA-DQ2 and simulations of its interaction with gluten peptides to explain molecular recognition in celiac disease. J. Mol. Graph. Model. 23, 419–431.

Cunha, F.Q., Moncada, S., Liew, F.Y., 1992. Interleukin-10 (IL-10) inhibits the induction of nitric oxide synthase by interferon-gamma in murine macrophages. Biochem. Biophys. Res. Commun. 182, 1155–1159.

de Waal Malefyt, R., Yssel, H., de Vries, J.E., 1993. Direct effects of IL-10 on subsets of human CD4+ T cell clones and resting T cells. Specific inhibition of IL-2 production and proliferation. J Immunol. 150, 4754–4765.

Ding, Y.Z., Fu, S., Zamarin, D., 2003. Interleukin-10, In: Thomson, A.W., Lotze, M.T. (Eds.), The Cytokine Handbook, 4th Edn. Academic Press, London, pp. 603–625.

Facchiano, A.M., Stiuso, P., Chiusano, M.L., Caraglia, M., Giuberti, G., Marra, M., Abruzzese, A., Colonna, G., 2001. Homology modelling of the human eukaryotic initiation factor 5A (eIF-5A). Protein Eng. 14, 881–890.

Facchiano, A.M., Costantini, S., Di Maro, A., Panichi, D., Chambery, A., Parente, A., Di Gennaro, S., Poerio, E., 2006. Modelling the 3D structure of WSCI. Probing the reactive site of the inhibitor with two susceptible proteinases, by time-course analysis and molecular dynamics simulations. Biol. Chem. 387, 931–940.

Felsenstein, J., 1985. Confidence limits on phylogenies: an approach using the bootstrap. Evolution 39, 783–791.

Fiorentino, D.F., Zlotnik, A., Mosmann, T.R., Howard, M., O'Garra, A., 1991. IL-10 inhibits cytokine production by activated macrophages. J. Immunol. 147, 3815–3822.

Gallagher, G., Dickensheets, H., Eskdale, J., Izotova, L.S., Mirochnitchenko, O.V., Peat, J.D., Vazquez, N., Pestka, S., Donnelly, R.P., Kotenko, S.V., 2000. Cloning, expression and initial characterisation of interleukin-19 (IL-19), a novel homologue of human interleukin-10 (IL-10). Genes Immun. 1, 442–450.

Gonzales, S.F., Huising, M.O., Stakauskas, R., Forlenza, M., Verburg-van Kemenade, B.M.L., Buchmann, K., Nielsen, M.E., Wiegertjes, G.F., 2007. Real-time expression analysis in carp (*Cyprinus carpio* L.) skin: inflammatory response to injury mimicking infection with ectoparasites. Dev. Comp. Immunol. 244–254.

Guex, N., Peitsch, M.C., 1997. SWISS-MODEL and the Swiss-PdbViewer: an environment for comparative protein modeling. Electrophoresis 18, 2714–2723.

Hubbard, S.J., Campbell, S.F., Thornton, J.M., 1991. Molecular recognition. Conformational analysis of limited proteolytic sites and serine proteinase protein inhibitors. J. Mol. Biol. 220, 507–530.

Inoue, Y., Kamota, S., Ito, K., Yoshiura, Y., Ototake, M., Moritomo, T., Nakanishi, T., 2005. Molecular cloning and expression analysis of rainbow trout (*Oncorhynchus mykiss*) interleukin-10 cDNAs. Fish Shellfish Immunol. 18, 335–344.

Jones, S., Thornton, J.M., 1996. Principles of protein–protein interactions derived from structural studies. Proc. Natl. Acad. Sci. 93, 13–20.

Please cite this article as: Buonocore, F. et al. Interleukin-10 expression by real-time PCR and homology modelling analysis in the European sea bass (*Dicentrarchus Labrax* L.). Aquaculture (2007), doi:10.1016/j.aquaculture.2007.05.040

Josephson, K., Logsdon, N.J., Walter, M.R., 2001. Crystal structure of the IL-10/IL-10R1 complex reveals a shared receptor binding site. Immunity 14, 35–46.

Julenius, K., Molgaard, A., Gupta, R., Brunak, S., 2005. Prediction, conservation analysis and structural characterization of mammalian mucin-type *O*-glycosylation sites. Glycobiology 15, 153–164.

Kabsch, W., Sander, C., 1983. Dictionary of protein secondary structure: pattern recognition of hydrogen-bonded and geometrical features. Biopolymers 22, 2577–2637.

Lagrelius, M., Jones, P., Franck, K., Gaines, H., 2006. Cytokine detection by multiplex technology useful for assessing antigen specific cytokine profiles and kinetics in whole blood cultured up to seven days. Cytokine 33, 156–165.

Laskowski, R.A., MacArthur, M.W., Moss, D.S., Thornton, J.M., 1993. PROCHECK — a program to check the stereochemical quality of protein structures. J. Appl. Crystallogr. 26, 283–291.

Marabotti, A., D'Auria, S., Rossi, M., Facchiano, A.M., 2004. Theoretical model of the three-dimensional structure of a sugar binding protein from *Pyrococcus horikoshii*: structural analysis and sugar binding simulations. Biochem. J. 280, 677–684.

Moore, K.W., O'Garra, A., de Waal Malefyt, R., Vieira, P., Mosmann, T.R., 1993. Interleukin-10. Annu. Rev. Immunol. 11, 165–190.

Moore, K.W., de Waal Malefyt, R., Coffman, R.L., O'Garra, A., 2001. Interleukin-10 and the interleukin-10 receptor. Annu. Rev. Immunol. 19, 683–765.

Mosmann, T.R., 1994. Properties and functions of interleukin-10. Adv. Immunol. 56, 1–26.

Mosmann, T.R., Moore, K.W., 1991. The role of IL-10 in cross-regulation of TH1 and TH2 responses. Immunol. Today 12, 49–53.

Nielsen, H., Engelbrecht, J., Brunak, S., von Heijne, G., 1997. Identification of prokaryotic and eukaryotic signal peptides and prediction of their cleavage sites. Protein Eng. 10, 1–6.

Orengo, C.A., Michie, A.D., Jones, S., Jones, D.T., Swindells, M.B., Thornton, J.M., 1997. CATH — a hierarchic classification of protein domain structures. Structure 5, 1093–1108.

Pearl, F.M.G., Lee, D., Bray, J.E., Sillitoe, I., Todd, A.E., Harrison, A.P., Thornton, J.M., Orengo, C.A., 2000. Assigning genomic sequences to CATH. Nucl. Acids Res. 28, 277–282.

Pearson, W.R., Lipman, D.J., 1988. Improved tools for biological sequence comparison. Proc. Natl. Acad. Sci. U.S.A. 85, 2444–2448.

Pinto, R.D., Nascimento, D.S., Reis, M.I.R., do Vale, A., dos Santos, N.M.S., 2006. Molecular characterisation, 3D modelling and expression analysis of sea bass (*Dicentrarchus labrax*) interleukin-10. Mol. Immunol. 44, 2066–2075.

Roncarolo, M.G., Gregori, S., Battaglia, M., Bacchetta, R., Fleischhauer, K., Levings, M.K., 2006. Interleukin-10-secreting type 1 regulatory T cells in rodents and mammals. Immunol. Rev. 212, 28–50.

Rothwell, L., Young, J.R., Zoorob, R., Whittaker, C.A., Hesketh, P., Archer, A., Smith, A.L., Kaiser, P., 2004. Cloning and characterization of chicken IL-10 and its role in the immune response to *Eimeria maxima*. J. Immunol. 173, 2675–2682.

Saitou, N., Nei, M., 1987. The Neighbour-Joining method — a new method for reconstructing phylogenetic trees. Mol. Biol. Evol. 4, 406–425.

Sali, A., Blundell, T.L., 1993. Comparative protein modelling by satisfaction of spatial restraints. J. Mol. Biol. 234, 779–815.

Savan, R., Igawa, D., Sakai, M., 2003. Cloning, characterization and expression analysis of interleukin-10 from the common carp, *Cyprinus carpio* L. Eur. J. Biochem. 270, 4647–4654.

Scapigliati, G., Buonocore, F., Bird, S., Zou, J., Pelegrin, P., Falasco, C., Prugnoli, D., Secombes, C.J., 2001. Phylogeny of cytokines: molecular cloning and expression analysis of sea bass *Dicentrarchus labrax* interleukin-1 beta. Fish Shellfish Immunol. 11, 711–726.

Scapigliati, G., Costantini, S., Colonna, G., Facchiano, A., Buonocore, F., Bossù, P., Holland, J.W., Secombes, C.J., 2004. Modelling of fish interleukin 1 and its receptor. Dev. Comp. Immunol. 28, 429–441.

Scapigliati, G., Buonocore, F., Mazzini, M., 2006a. Biological activity of cytokine: an evolutionary perspective. Curr. Pharm. Des. 12, 3071–3081.

Scapigliati, G., Mazzini, M., Buonocore, F., 2006b. Biological activity of cytokines: an evolutionary perspective. Curr. Pharm. Design 12, 3071–3081.

Sippl, M.J., 1993. Recognition of errors in three-dimensional structures of proteins. Proteins 17, 355–362.

Street, N.E., Mosmann, T.R., 1991. Functional diversity of T lymphocytes due to secretion of different cytokine patterns. FASEB J. 5, 171–177.

Tan, J.C., Indelicato, S.R., Narula, S.K., Zavodny, P.J., Chou, C.C., 1993. Characterization of interleukin-10 receptors on human and mouse cells. J. Biol. Chem. 268, 21053–21059.

Thompson, J.D., Higgins, D.G., Gibson, T.J., 1994. Clustal W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucl. Acids Res. 22, 4673–4680.

Wallner, B., Elofsson, A., 2005. All are not equal: a benchmark of different homology modeling programs. Protein Sci. 14, 1315–1327.

Windsor, W.T., Syto, R., Tsarbopoulos, A., Zhang, R., Durkin, J., Baldwin, S., Paliwal, S., Mui, P.W., Pramanik, B., Trotta, P.P., et al., 1993. Disulfide bond assignments and secondary structure analysis of human and murine interleukin-10. Biochemistry 32, 8807–88015.

Zdanov, A., Schalk-Hihi, C., Gustchina, A., Tsang, M., Weatherbee, J., Wlodawer, A., 1995. Crystal structure of interleukin-10 reveals the functional dimer with an unexpected topological similarity to interferon gamma. Structure 3, 591–601.

Zhang, D.C., Shao, Y.Q., Huang, Y.Q., Jiang, S.G., 2005. Cloning, characterization and expression analysis of interleukin-10 from the zebrafish (*Danio rerio*). J. Biochem. Mol. Biol. 38, 571–576.

Zou, J., Clark, M.S., Secombes, C.J., 2003. Characterisation, expression and promoter analysis of an interleukin 10 homologue in the puffer fish, *Fugu rubripes*. Immunogenetics 55, 325–335.

Please cite this article as: Buonocore, F. et al. Interleukin-10 expression by real-time PCR and homology modelling analysis in the European sea bass (*Dicentrarchus Labrax* L.). Aquaculture (2007), doi:10.1016/j.aquaculture.2007.05.040